

# Analysis of Progressive Deletions of the Transmembrane and Cytoplasmic Domains of Influenza Hemagglutinin

RS995.5

Carolyn Doyle,\* Joe Sambrook,‡ and Mary-Jane Gething‡§

\*Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724; ‡Department of Biochemistry and §Howard Hughes Medical Institute, University of Texas Health Science Center, Dallas, Texas 75235. Dr. Doyle's present address is Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138.

NOTICE: This Material  
may be protected by copyright  
law. (Title 17 US. Code)

**Abstract.** Site-directed oligonucleotide mutagenesis has been used to introduce chain termination codons into the cloned DNA sequences encoding the carboxy-terminal transmembrane (27 amino acids) and cytoplasmic (10 amino acids) domains of influenza virus hemagglutinin (HA). Four mutant genes were constructed which express truncated forms of HA that lack the cytoplasmic domain and terminate at amino acids 9, 14, 17, or 27 of the wild-type hydrophobic

domain. Analysis of the biosynthesis and intracellular transport of these mutants shows that (a) the cytoplasmic tail is not needed for the efficient transport of HA to the cell surface; (b) the stop-transfer sequences are located in the hydrophobic domain; (c) 17 hydrophobic amino acids are sufficient to anchor HA stably in the membrane; and (d) mutant proteins with truncated hydrophobic domains show drastic alterations in transport, membrane association, and stability.

**G**LYCOPROTEINS that span the plasma membrane once typically contain a stretch of 20–25 consecutive uncharged residues with considerable hydrophobic character (41, 45, 50, 54), followed by a sequence of hydrophilic amino acids that varies both in amino acid sequence and length and lies on the cytoplasmic side of the lipid bilayer. The functions of these two domains have been investigated in a number of laboratories by analyzing the biosynthesis, intracellular transport, and biological properties of mutant and chimeric forms of a number of different integral membrane proteins. Deletion of the DNA sequences encoding the transmembrane and cytoplasmic tail domains of influenza virus hemagglutinin (HA)<sup>1</sup> (17, 51), vesicular stomatitis virus (VSV) G protein (42), herpes simplex glycoprotein D (27), or a murine Class I histocompatibility antigen (64) resulted in the expression of truncated forms of the proteins that were secreted from eukaryotic cells. In other studies, deletion of the transmembrane domain resulted in both loss of membrane association and an inability of the mutant glycoprotein to be transported from the endoplasmic reticulum (39, 56, 57). In a prokaryotic system, deletion of the transmembrane domain converted the coliphage f1 gene III protein from an integral membrane protein to a secreted periplasmic form (9). Membrane association of many of these truncated proteins could be restored by addition of authentic transmembrane domains from other integral membrane proteins (18, 38, 44) or addition of artificial domains of 16 or more hydrophobic amino acids (8). Furthermore,

conversion of a secretory protein to a membrane-bound form has been achieved by fusing the transmembrane and cytoplasmic domains of VSV G protein or influenza HA to rat growth hormone (20, 40). Finally, expression in a cell-free system of a chimeric protein consisting of the transmembrane anchor of an IgM heavy chain inserted between  $\beta$ -lactamase and  $\alpha$ -globin resulted in a transmembrane protein with the predicted topology (60). These results have established that a major function of the carboxy-terminal hydrophobic domain is to anchor the transmembrane protein in the lipid bilayer. Alteration or partial deletion of cytoplasmic tails (6, 7, 12, 14, 32, 37, 57, 63, 64) does not usually affect membrane integration although the transport of the resulting mutant proteins through the secretory pathway may be inhibited, probably as a consequence of protein denaturation or mal-folding (12, 18a).

Correct positioning of an integral membrane protein in the lipid bilayer requires some form of stop-transfer signal that halts the co-translational translocation of the nascent polypeptide across the membrane of the rough endoplasmic reticulum (45). Although the nature of such signals has not been unambiguously established, it has been proposed that the hydrophobic transmembrane domain and the positively charged amino acid that frequently marks the beginning of the hydrophilic cytoplasmic domain may act together to halt the translocation process (4, 45). Recent analyses of membrane proteins with mutated cytoplasmic domains indicate that positively charged residues may not play an obligatory role in stop-transfer (6, 64).

In this paper we have investigated the function of the carboxy-terminal hydrophobic and cytoplasmic domains of HA by site-directed oligonucleotide mutagenesis of the DNA

1. Abbreviations used in this paper: endo H, endoglycosidase H; HA, hemagglutinin; VSV, vesicular stomatitis virus.

BEST AVAILABLE COPY

sequence coding for these regions of the protein. We have introduced translational termination codons at four sites to generate mutant forms of HA that are 10, 20, 23, and 28 amino acids shorter than the wild-type protein. All four of these mutant proteins lack the entire sequences of the cytoplasmic tail but retain variable quantities of the hydrophobic transmembrane region. Analysis of the biosynthesis and intracellular transport of the mutants shows that (a) the cytoplasmic domain of HA contains no signals that are essential for movement of the protein to the cell surface; (b) the stop-transfer signal is located within the hydrophobic transmembrane domain; (c) the entire hydrophobic region is not needed to anchor HA in the membrane; and (d) severe truncation of the hydrophobic domain affects the membrane association, stability, and intracellular transport of HA.

## Materials and Methods

### General Recombinant DNA Techniques

Buffers and reaction conditions for restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were those listed by the commercial source, New England Biolabs (Beverly, MA). Reverse transcriptase was obtained from Life Sciences Inc. (St. Petersburg, FL), and RNasin was obtained from Promega Biotech (Madison, WI). Isolation of DNA fragments, addition of restriction site linkers, preparation of plasmid DNAs, and other standard recombinant DNA techniques were carried out as described in Maniatis et al. (30) and in other references cited therein. Transformation of *Escherichia coli* DH-1 or TG-1 cells was done by the method of Hanahan (21).

### Construction and Cloning of a Full-length cDNA of the HA from the X-31 Strain of Influenza Virus

X-31, a recombinant influenza virus bearing the HA from the A/Aichi/68 strain (25) was kindly supplied by John Skehel, National Institute of Medical Research, Mill Hill, United Kingdom. Purification of virion RNAs and the construction and cDNA cloning of the nucleotide sequences encoding the ectodomain of the X-31 HA have been described previously (11). To synthesize the full-length gene, a 316-bp XbaI-BamHI restriction fragment (nucleotides 1,302-1,618) was isolated from the 3' end of the partial cDNA clone. This fragment was annealed to total Aichi virion RNAs and double-stranded cDNA synthesis was carried out according to published procedures (30). After second strand synthesis, the 3'-terminal hairpin loop was excised using S1 nuclease and the DNA ends were repaired using the Klenow fragment of DNA polymerase I before ligation to phosphorylated Sall linkers. The double-stranded DNA molecules were then cleaved with BamHI and Sall to yield fragments of ~110 bp which were inserted into the M13 cloning vector, mp18 (31, 47). Single-stranded DNAs were prepared from single plaque isolates and clones (mp18-HA-TMT) containing the complete carboxy-terminal coding region were identified by DNA sequence analysis using the chain termination technique (46). The double-stranded replicative form of the M13 DNA was prepared from phage containing this complete sequence. The 112-bp BamHI-Sall restriction fragment encoding the transmembrane and cytoplasmic domains of X-31 HA was isolated and inserted into an SV40 vector downstream of the sequences encoding the X-31 HA ectodomain to generate a full length cDNA copy of the HA gene (see below and Fig. 1A).

### Site-specific Mutagenesis of HAcDNA Using Mismatched Oligonucleotides

Four 17-mer oligonucleotides that correspond except for a single mismatch to sequences encoding regions of the transmembrane domain and cytoplasmic tail of X-31 HA were used to generate single base substitutions that introduced termination codons into the HA gene (Fig. 2). The oligonucleotides were synthesized using an Applied Biosystems 380-A DNA Synthesizer and purified by electrophoresis through 20% polyacrylamide gels containing 7 M urea. Mutagenesis was carried out by the double-primer method of Zoller and Smith (61, 62) using mp18-HA-TMT as the template. As described in Fig. 1B, the oligonucleotide primers were extended using the Klenow fragment of DNA polymerase I in the presence of T4 DNA li-

gase. Competent TG-1 cells were transfected with an aliquot of the reaction mixture which contained partially and fully double-stranded DNA molecules. The resulting plaques were screened by *in situ* hybridization using the appropriate <sup>32</sup>P-labeled mutagenic oligonucleotides as probe. Bacteriophages containing mutant sequences were plaque purified, and single-stranded DNA was purified for use as template for sequence analysis by the chain termination method of Sanger et al. (46, 47). Once the desired base substitution had been confirmed, the double-stranded replicative form of each mutant phage DNA was prepared and purified by centrifugation on CsCl<sub>2</sub> gradients. The BamHI-Sall HA DNA fragment carrying each point mutation was isolated and used to replace the equivalent wild-type sequences in the SVEXHA recombinant viral genome (see below and Fig. 1B).

### SV40 Vectors Designed To Express Wild-type and Truncated Forms of X-31 HA

A vector, SVEXHA-A<sup>-</sup> (Fig. 1A), has previously been constructed to express the X-31 HA ectodomain (Bye, J., and M. J. Gething, unpublished results). This vector closely resembles SVEHA20-A<sup>-</sup> which has been used to express the ectodomain of the HA from the A/Japan/305/57 strain of influenza virus (17). pSVEXHA-A<sup>-</sup> contains the ClaI-BamHI restriction fragment encoding the X-31 HA ectodomain (11) inserted between the HpaII (nucleotide 346) and BamHI (nucleotide 2533) sites of SV40 DNA, so that the HA sequences replace the late region of the SV40 genome, which encodes the capsid proteins. For amplification and manipulation of the DNA sequences, the genome was inserted through the unique KpnI site in the SV40 sequence into plasmid pKSB (12). The construction of a vector that contains the full length copy of the wild-type X-31 HA gene is shown in Fig. 1A. Plasmid pSVEXHA-A<sup>-</sup> was cleaved with BamHI, and a BamHI-Sall-XbaII adaptor linker was inserted into the BamHI site. The 112-bp BamHI-Sall fragment encoding the carboxy-terminal sequences of HA (see above) was then inserted between the new BamHI and Sall sites of the vector to yield pSVEXHA. For expression of the truncated, mutant forms of HA, the 112-bp BamHI-Sall fragments containing new termination codons introduced by oligonucleotide-directed mutagenesis were inserted into the pSVEXHA vector, replacing the wild-type fragment (see above and Fig. 1B). Before generation of recombinant virus stocks, the pKSB plasmid sequences were removed by digestion with KpnI, and the recombinant SV40-HA genome was purified by gel electrophoresis and recircularized by dilute ligation (16).

### Generation of SV40-HA Recombinant Virus Stocks and Infection of CV-1 Cells

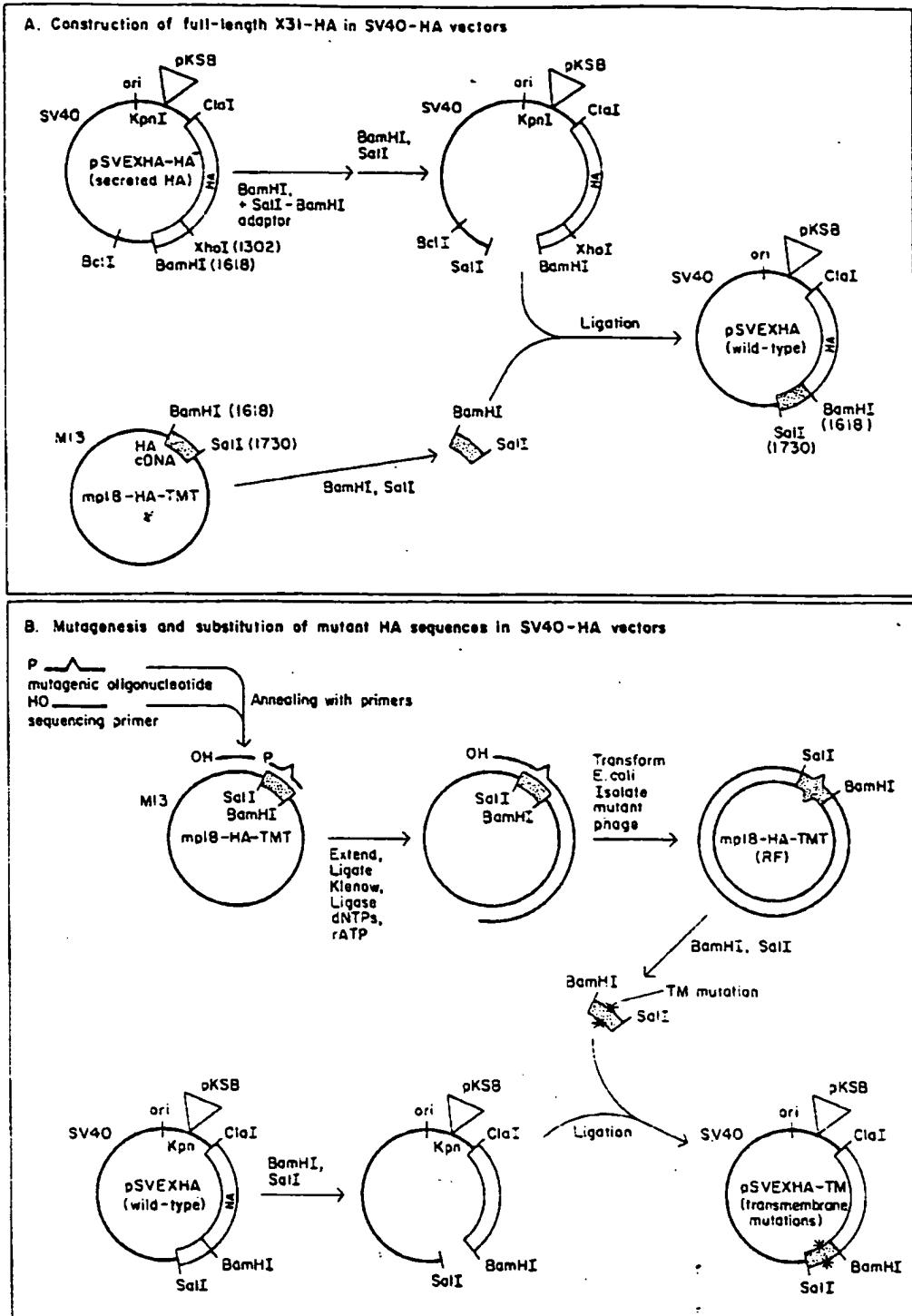
SV40-HA recombinant genomes containing wild-type and mutant forms of the X-31 HA gene were transfected into CV-1 cells using DEAE-dextran and chloroquine as previously described (12). High-titer virus stocks were developed and used to infect fresh monolayers of CV-1 cells for analysis of the biosynthesis and intracellular transport of wild-type and mutant HAs (16). Conditions for growth and infection of CV-1 cells were as described previously (11).

### Analysis of the Biosynthesis and Intracellular Processing of Wild-type and Mutant HAs

To quantitate the amount of HA protein produced, solid-phase radioimmunoassay of supernatants and extracts of CV-1 cells infected with SVEXHA recombinant viruses was carried out as described previously (11, 16) using a high titer rabbit antiserum directed against X-31 HA. Analysis of the biosynthesis of wild-type and mutant HA proteins was carried out by pulse-chase radiolabeling of infected cells with [<sup>35</sup>S]methionine followed by immunoprecipitation and SDS PAGE (17). Digestion with endoglycosidase H (endo H) was carried out as described by Owen et al. (35). Binding of red cells to hemagglutinin expressed on the surface of infected CV-1 cells was performed as described previously (16). The location of wild-type and mutant forms of HA was determined by indirect immunofluorescence of infected cells (12).

### Phase Partitioning of Wild-type and Mutant HAs in Triton X-114 Solutions

36 h after infection with SVEXHA recombinant viruses, monolayers of CV-1 cells were washed with ice cold Tris-buffered saline and then extracted on ice in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, containing 1% Triton X-114. After removal of nuclei and cell debris by brief centrifugation, the cell extract was separated into detergent and aqueous (pellet and supernatant) phases as described by Bordier (5). The detergent phase was recon-



**Figure 1.** Mutagenesis and expression of X-31 HA. (A) Construction of an SV40-based recombinant vector (SVEXHA) to express a full-length copy of the cDNA encoding X-31 HA. (B) Oligonucleotide-directed mutagenesis of sequences encoding the transmembrane and cytoplasmic domains of X-31 HA and construction of SVEXHA-TM recombinant vectors to express truncated HA proteins. Details of the procedures used are described in Materials and Methods.

stituted to the same volume as the aqueous phase by addition of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and equal aliquots of the two phases were immunoprecipitated using anti-X31 HA antiserum. The amount of HA protein in each phase was visualized by separation of the immunoprecipitated proteins by SDS PAGE and autoradiography.

## Results

### Construction and Expression of Mutant Genes Coding for HAs with Altered Transmembrane and Cytoplasmic Domains

Site-directed oligonucleotide mutagenesis was used to in-

duce chain termination codons at four sites within the DNA sequences coding for the transmembrane and cytoplasmic tail regions of the HA from the X-31 strain of influenza virus (see Materials and Methods and Fig. 1). The locations of these mutations are shown in Fig. 2. In mutant HA-TM27, UGA replaces the AGA triplet (Arg) at codon 27 of the hydrophobic transmembrane sequence, resulting in the synthesis of a truncated protein that terminates exactly at the end of the transmembrane region and lacks all sequences of the hydrophilic cytoplasmic tail. In mutants HA-TM17, HA-TM14, and HA-TM9, termination codons have been substituted for UUG (Leu), UGU (Cys), and UCA (Ser) at

coding strand	... ... Trp Ile Leu Trp Ile Ser Phe
non-coding strand	... ... TCG ATC CTG TGG ATT TCC TTT
	... ... ACC TAG GAC ACC TAA AGG AAA

Ala Ile Ser Cys Phe Leu Leu Cys Val Val Leu Leu Gly Phe Ile Met	... ... TCG ATC CTG TGG ATT TCC TTT
GCC ATA TCA TGC TTT TTG CTT TGT GTT TTG CTG GGC TTC ATC ATG	... ... ACC TAG GAC ACC TAA AGG AAA
CGG TAT AGT ACG AAA AAC GAA ACA CAA AAC GAC CCC AAG TAG TAC	
(tm14) CTT TGA GTT GTT TTG CT	
(tm9) C ATA TGA TGC TTT TTG C	(tm17) T GTT TAG CTG GGG TTC A

Trp Ala Cys Gln Arg Gly Asn Ile Arg Cys Asn Ile Cys Ile STOP	... ... TCG ATC CTG TGG ATT TCC TTT
TGG GCC TGC CAG AGA GGC AAC ATT AGG TGC AAC AAT TGC ATT TGA	... ... ACC TAG GAC ACC TAA AGG AAA
ACC CGG ACG GTC TCT CCG TTG TAA TCC ACG TTG TTA ACC TAA ACT	
(tm27) GC CAG TGA GGC AAC ATT	

amino acid positions 17, 14, and 9, respectively, within the hydrophobic sequence. These mutants express forms of HA that lack both the cytoplasmic tail and progressively increasing portions of the hydrophobic domain (Fig. 3).

SV40-HA recombinant genomes were constructed in which either the wild-type HA gene (XHA) or the mutant genes (HA-TM27, HA-TM17, HA-TM14, and HA-TM9) were used to replace the coding sequences of the late region of SV40 DNA (see Materials and Methods and Fig. 1). These recombinant genomes were transfected into simian CV-1 cells and high titer recombinant virus stocks were developed as described previously (12, 16). Monolayers of CV-1 cells were infected with these virus stocks and the amount of HA present in supernatants and cell extracts was measured by solid-phase radioimmune assay. After 60 h, cells infected with SVEXHA, SVHA-TM27, and SVHA-TM17 contained levels of X-31 HA similar to those obtained in this expression system for the HA of the Japan strain of influenza virus (16), i.e.,  $\sim 10^8$  molecules of HA per cell. By contrast, CV-1 cells infected with SVHA-TM14 and SVHA-TM9 contained  $\sim 5$ -10-fold fewer HA molecules per cell. Data presented below (see Fig. 5) show that although the wild-type HA and all four mutants were synthesized at approximately the same rate, the HAs of TM-14 and TM-9 were less stable than those of wild-type, TM-27, or TM-17. Thus less HA accumulated during the course of infection of CV-1 cells with these mutants; it is this accumulation of the protein that is measured by the radioimmune assay. In no case was any HA antigen detected in the medium of infected cells indicating that truncation of the hydrophobic anchor of HA to a length as short as nine hydrophobic amino acids did not result in secretion of the protein from the cell.

#### *Cellular Localization of Truncated Forms of HA*

Indirect immunofluorescence and hemagglutination assays were used to determine the cellular location of truncated forms of HA in infected CV-1 cells. Fig. 4 shows examples of the results obtained when cells infected for 42 h with the various recombinant viruses were assayed for erythrocyte binding or were permeabilized and stained with an HA-specific antisera to reveal both intracellular and cell surface forms of the wild-type and truncated HAs. Cells infected with SVHA-TM27 displayed patterns of hemagglutination

**Figure 2.** Oligonucleotide-directed mutagenesis of sequences encoding the transmembrane and cytoplasmic domains of X-31 HA. The nucleotide sequence of the DNA encoding the transmembrane and cytoplasmic domains of X-31 HA is shown together with the mismatched 17-mer oligonucleotides used to introduce termination codons within the membrane-spanning region.

and immunofluorescent labeling that were indistinguishable from those seen in CV-1 cells infected with the wild-type SVEXHA recombinant vector. Guinea pig erythrocytes bound densely to  $>90$ –95% of the cells expressing X-31 HA or HA-TM27. In addition to diffuse fluorescent labeling of the cell surface, there was strong staining of the perinuclear region as well as reticular staining of the cytoplasm—a pattern that is typical of glycoproteins synthesized in the endoplasmic reticulum and transported to the cell surface via the Golgi apparatus (12, 16, 17).

By contrast, only 5–10% of the cells expressing mutant HA-TM17 bound erythrocytes or displayed any surface fluorescence. These results were obtained consistently, even though  $>95$ % of the cells were shown to be infected by using for immunofluorescence an antiserum which recognizes SV40 T-antigen. At late times (60–72 h) in the infection, the proportion of cells expressing detectable amounts of surface HA increased to a maximum of 15–20% of the total population. Those cells that showed no surface fluorescence displayed very strong staining of the endoplasmic reticulum. In some cells, there also appeared to be a concentration of fluorescence in the juxtanuclear region, which might be taken as evidence that the HA proteins were also located within the Golgi stacks. However, similar patterns of staining were seen in control experiments in which infected CV-1 cells were stained with an antiserum (29) that reacts specifically with antigens localized only in the endoplasmic reticulum. Furthermore, Poruchynsky et al. (36) have shown that in COS-7 cells (19), which are derived from the CV-1 cells used in this study, the Golgi stacks in the juxtanuclear region are spatially intertwined with an extensive endoplasmic reticulum, so that in this region bright but not totally coincident immunofluorescence is obtained with probes for either endoplasmic reticulum or Golgi elements. The immunofluorescent staining pattern of cells expressing HA-TM17, taken together with the results of the analysis of the biosynthesis of this molecule (see below), suggest that the transport of HA-TM17 along the exocytotic pathway is defective. In most cells of the infected population the mutant protein did not progress through the Golgi apparatus; however in a small proportion of cells, the protein reached the plasma membrane at a concentration sufficient for its display in a biologically active form. The cause of this heterogeneity is unknown, but it has been observed previously in cells ex-

	Transmembrane Domain	Cytoplasmic Tail
HA-X31-WT	...D WILWISFAISCFLLCVVLLGFIMWACQ	RGNIRCNICI
HA-TM27	...D WILWISFAISCFLLCVVLLGFIMWACQ	
HA-TM17	...D WILWISFAISCFLLCVV	
HA-TM14	...D WILWISFAISCFLL	
HA-TM9	...D WILWISFAI	
HA-A	...D WIQT	

Figure 3. Comparison of the carboxyl-terminal sequences of the wild-type and truncated X-31 HA proteins.

pressing another mutant of HA (HA164) that is blocked at a later, post-Golgi stage in the exocytic pathway (12).

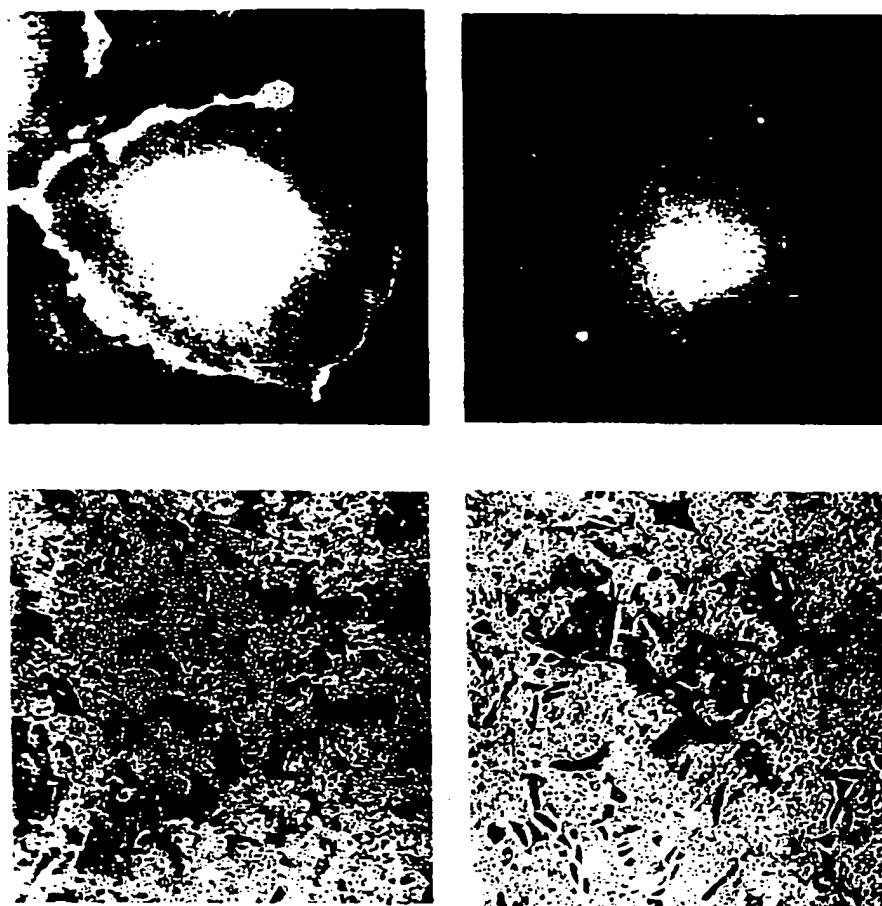
Cells expressing HA-TM14 and HA-TM9 were unable to bind erythrocytes at any stage during the infectious cycle. The infected cells displayed a pattern of immunofluorescent staining consistent with localization of the mutant proteins solely in the endoplasmic reticulum. Furthermore, the staining pattern seen in cells infected with SVHA-TM14 or SVHA-TM9 was identical to that seen in cells expressing another mutant of HA (HAxpBR) which has been shown previously to be restricted to the endoplasmic reticulum (12). These data suggest that HA-TM14 and HA-TM9 are blocked at an early stage in the transport pathway and are unable to move to the Golgi complex. Striking differences are apparent between the pattern of fluorescence displayed by cells infected by HA-TM17 or by HA-TM14 or HA-TM9 (Fig. 4) even though the biochemical data indicate that all three mutant proteins are transported inefficiently to the Golgi complex. The reticular pattern of HA-TM17 is much more extensive, reaching almost to the periphery of the cell. By contrast, HA-TM14 and HA-TM9 molecules appear to be localized closer to the nucleus. It remains to be established whether this apparent difference in intracellular localization is merely a consequence of the presence of greater numbers of HA-TM17 molecules in the endoplasmic reticulum, or whether this protein progresses further than HA-TM14 or HA-TM9 along the exocytic pathway, for example to the smooth endoplasmic reticulum or to a pre-Golgi reticulum such as that described by Saraste and Kuismannen (48).

#### *Biochemical Analysis of the Transport of Truncated Forms of HA*

The synthesis and processing of the mutant glycoproteins was examined by immunoprecipitation of lysates of CV-1 cells that had been infected for 42 h with the various SVEXHA vectors and then pulse-labeled with [<sup>35</sup>S]methionine. In all cases, when cells infected with the mutant viruses were pulse-labeled for 10 min, the immunoprecipitated HA polypeptides migrated through SDS polyacrylamide gels with an apparent molecular mass of ~74–76 kD (Fig. 5). The molecular mass of the nonglycosylated forms of both wild-type and mutant HAs synthesized in tunicamycin-treated

cells is ~63 kD (data not shown). Thus, all of the mutant proteins acquired core oligosaccharide units in the endoplasmic reticulum.

Trimming of mannose-rich core oligosaccharides begins in the endoplasmic reticulum and is completed after the glycoprotein arrives in the medial portion of the Golgi apparatus (26). Mannose-rich core oligosaccharides can be removed from the polypeptide(s) in vitro by digestion with endo H (53), while completely trimmed core oligosaccharides are resistant to the enzyme. Thus, acquisition of resistance to endo H is a reliable measure of transport of glycoproteins from the endoplasmic reticulum to the medial Golgi apparatus. To determine how truncation of the transmembrane domain of the HA might affect its transport, CV-1 cells infected with recombinant viruses were pulse-labeled with [<sup>35</sup>S]methionine for 10 min and incubated in the presence of an excess of nonradioactive methionine for periods up to 8 h. Aliquots of the immunoprecipitated HAs were treated with endo H and analyzed by SDS PAGE. The results of these experiments are shown in Fig. 5. The wild-type X-31 HA and HA-TM27 molecules synthesized in the 10-min labeling period became largely resistant to the enzyme during a 30-min chase and maximally resistant during 1 h of chase. X-31 HA has seven potential glycosylation sites and complete removal of core oligosaccharides from these sites reduces the molecular mass of the protein by ~8,000 D (see Fig. 5). If all the core oligosaccharides were completely trimmed, digestion with endo H should have no effect on the molecular mass of the protein. However, even after extended periods of chase when the wild-type or TM-27 HAs had gained maximal resistance to endo H, digestion with the enzyme reduced their molecular mass by ~2,500 D. This is a consequence of the mature X-31 HA bearing both simple and complex glycans: normally only 5 of the 7 core oligosaccharides originally added to the protein in the endoplasmic reticulum are trimmed and processed—the oligosaccharides added at the two other sites always remain sensitive to digestion with endo H even after the protein has passed completely through the Golgi apparatus (33). Analysis of the three-dimensional structure of X-31 HA has shown that these carbohydrate side-chains are located in clefts of the molecule, where they are probably shielded from trimming and processing enzymes (58).



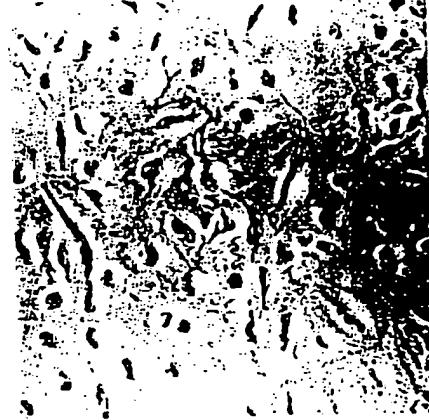
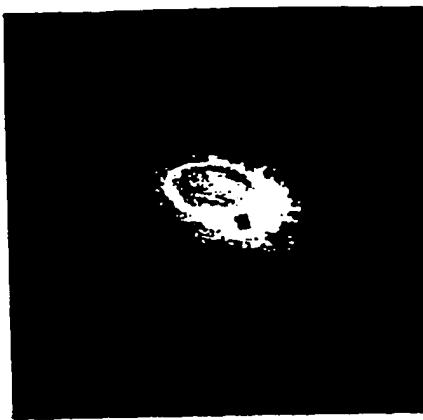
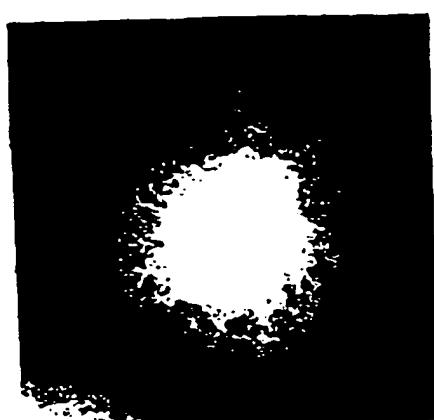
**Figure 4.** Intracellular localization of wild-type and truncated X-31 HAs expressed in CV-1 cells. 42 h after infection with SVEXHA or SVEXHA-TM vectors, CV-1 cells were assayed for localization of HA by indirect immunofluorescence using specific anti-X31 HA serum (top) or for surface expression of HA by hemagglutination of guinea pig erythrocytes (bottom).

The acquisition of resistance to endo H by HA-TM17 was significantly delayed in comparison to the wild-type protein. Endo H-resistant material was detectable only after 4 h of chase, and only when the autoradiogram was exposed for a very long period of time (such an exposure is shown in Fig. 6). Thus, only a small amount of newly synthesized glycoprotein (<5% of the  $^{35}$ S-labeled protein) became terminally glycosylated (indicated by arrows in Fig. 5). These data are consistent with the results obtained from immunofluorescent analysis of infected cells: the transport of the majority of HA-TM17 molecules between the endoplasmic reticulum and the medial Golgi is blocked or severely delayed. The small proportion of radiolabeled HA-TM17 that became resistant to endo H is probably the source of the material detected by immunofluorescence on the plasma membrane of 5–10% of the infected cell population. This material had a slightly higher molecular mass than mature X-31 HA or HA-TM27 and no reduction in its size occurred after digestion with endo H. It therefore appears that all seven of the oligosaccharides on a small fraction of HA-TM17 molecules became terminally glycosylated. A possible explanation for this finding would be that HA-TM17 folds much more slowly than wild-type X-31 HA thereby allowing the two mannose-rich oligosaccharides that normally are shielded to remain exposed long enough for them to be completely processed.

When cells infected with SVHA-TM14 and SVHA-TM9

were pulse-labeled with [ $^{35}$ S]methionine and then incubated with an excess of nonradioactive methionine, the amounts of immunoprecipitable HA decreased with increasing length of chase. Pulse-labeled HA-TM14 protein began to disappear after 6–8 h of chase (data not shown), while radiolabeled HA-TM9 was completely absent from extracts of infected cells after 2 h of chase (see Fig. 5). Both forms of HA remained completely sensitive to digestion with endo H for as long as they were detectable. These results are consistent with the observation (Fig. 4) that HA-TM14 and HA-TM9 were not transported beyond the endoplasmic reticulum or a pre-Golgi compartment in the exocytic pathway. Furthermore, their relative instability accounts for the comparatively small amounts of these truncated forms of HA that accumulated during the course of infection of CV-1 cells.

To measure the efficiency with which the various truncated forms of HA reached the cell surface, infected cells were pulse-labeled with [ $^{35}$ S]methionine and then incubated for different lengths of time in serum-free medium containing an excess of nonradioactive methionine. For the last 15 min of the chase period, trypsin was added to the medium at a concentration (5  $\mu$ g/ml) sufficient to cleave any HA at the cell surface into its component HA1 and HA2 subunits (15). The radiolabeled proteins were immunoprecipitated from cell extracts with anti-HA serum and separated by SDS PAGE. In agreement with previously published data (12, 17, 44), all of



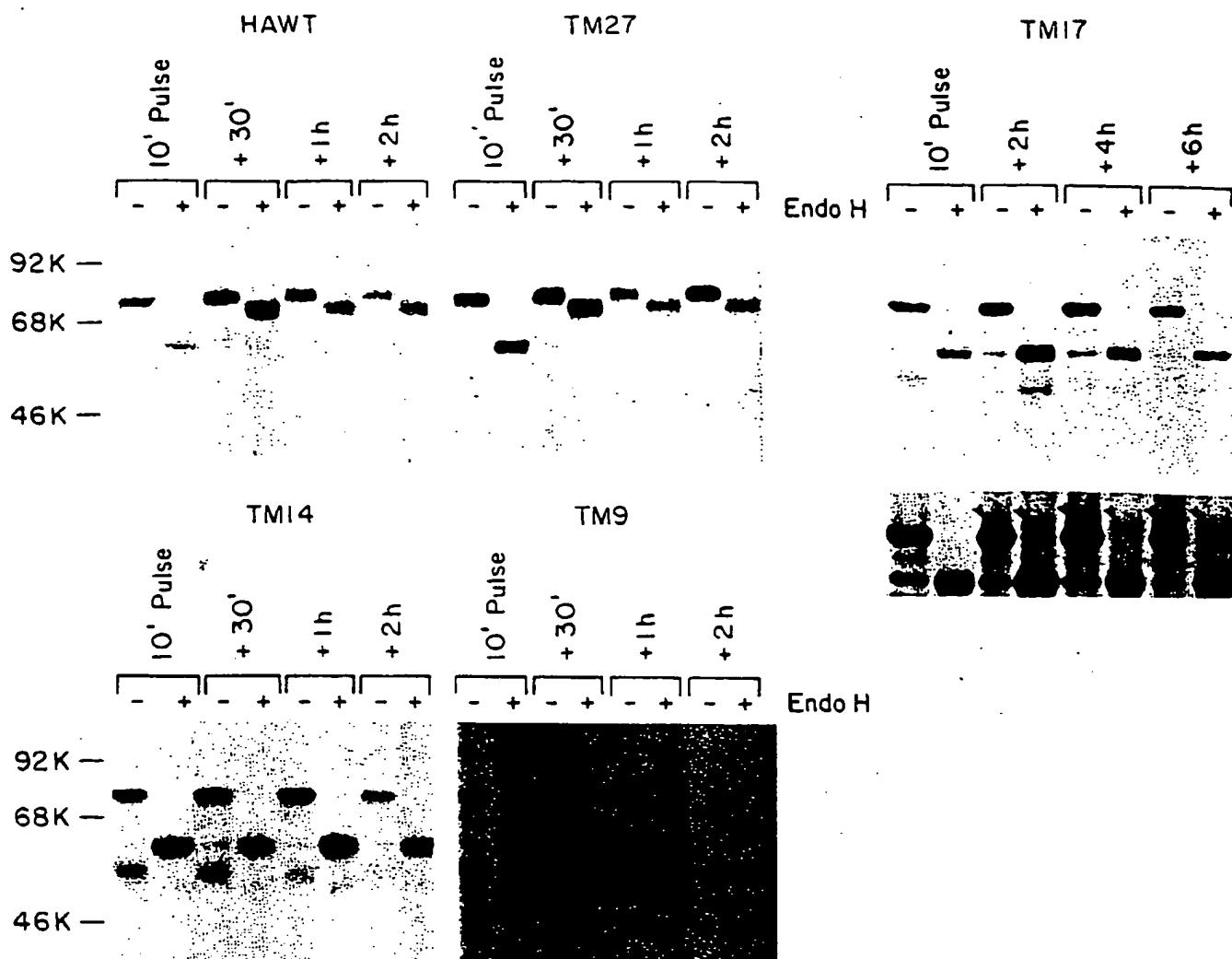
the radiolabeled wild-type protein became accessible to exogenously added trypsin within 2 h of chase (Fig. 6). HA-TM27 reached the cell surface with kinetics that were indistinguishable from those of the wild-type protein (results not shown). However, only a small proportion of radiolabeled HA-TM17 became accessible to cleavage by trypsin (~5% after 4 h of chase and ~10% after 6 h [see Fig. 6]). Comparison of the rate at which HA-TM17 became resistant to endo H with its course of appearance at the cell surface indicates that the delay in transport of the truncated protein occurs primarily between the endoplasmic reticulum and the Golgi apparatus. Even though the rate of transport of HA-TM17 was very slow, the protein gradually accumulated on the cell surface during the course of infection, eventually in some cells attaining a level sufficient for hemadsorption and for detection by immunofluorescence (Fig. 4). The molecules of HA-TM17 which reached the plasma membrane were biologically active and showed no sign of instability.

By contrast, HA-TM14 or HA-TM9 in intact cells always remained resistant to trypsin added in the extracellular medium (results not shown). This result is consistent with our inability to detect these proteins at the cell surface by the other assays described above. As seen previously, diminishing amounts of radiolabeled mutant proteins were immunoprecipitated from extracts of infected cells as the length of the chase was increased. We conclude that extensive truncation

of the transmembrane domain severely compromises the ability of HA to travel along the exocytic pathway and renders it susceptible to proteolytic destruction.

#### Triton X-114 Partitioning of the Truncated Forms of HA

The partitioning of proteins between an aqueous phase and Triton X-114 is used as a measure of their surface hydrophobicity and in particular as a reflection of the interaction of membrane proteins with the lipid bilayer (3, 5). To determine whether removal of the cytoplasmic tail and truncation of the transmembrane anchor of X-31 HA altered the detergent partitioning of the protein, an analysis of the partitioning of the wild-type and truncated forms of X-31 HA into TX-114 was performed. CV-1 cells infected for 42 h with the various SVEXHA recombinant vectors were pulse-labeled for 10 min with [<sup>35</sup>S]methionine or pulse-labeled and then incubated for a further 60 min in the presence of nonradioactive methionine before extraction with buffer containing 1% TX-114. The cell extracts were then partitioned as described in Materials and Methods, and the aqueous supernatant and detergent pellet fractions were immunoprecipitated and analyzed by SDS PAGE. The results shown in Fig. 7 show that newly synthesized wild-type X-31 HA was distributed approximately equally between the aqueous and detergent phases; however, partitioning into the TX-114 phase became



**Figure 5.** SDS PAGE of X-31 HA and mutant HA-TM proteins. CV-1 cells infected for 42 h with either SVEXHA or SVEXHA-TM recombinant virus stocks were labeled for 10 min with [ $^{35}$ S]methionine and then incubated in medium containing an excess of nonradioactive methionine for the times shown. Cell extracts were prepared, and proteins were immunoprecipitated with anti-HA serum. Aliquots of the precipitated proteins were treated in the presence or absence of endo H and then separated by SDS PAGE and autoradiographed. Below the panel showing the results for mutant TM17 is a darker exposure of a portion of the same autoradiogram, with arrows indicating processed material.

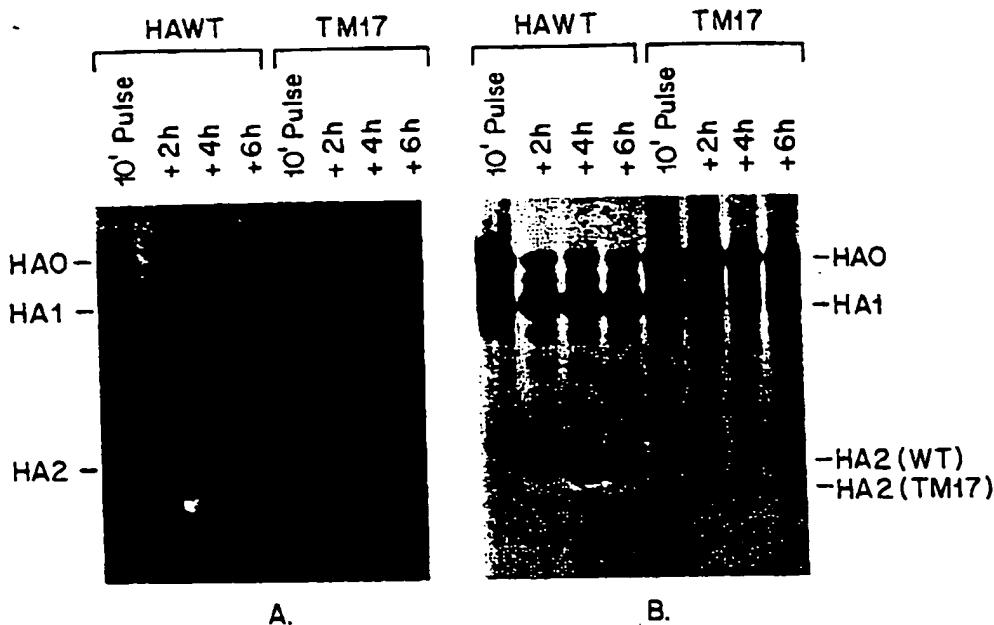
almost complete during the chase period. In other studies (18a), we have correlated the time course of partitioning of HA into TX-114 (and the stable integration of the protein in the lipid bilayer) with the time course of folding and trimerization of the HA molecule.

By contrast, the anchor-minus form of the protein, which lacked all of the hydrophobic amino acids of the transmembrane region, always remained in the aqueous phase. This was the case for both the core and terminally glycosylated forms of the protein located within the cell, and for the terminally glycosylated protein that after a 1-h chase period had been secreted into the medium above the cells. The partitioning of HA-TM27 into the detergent phase was similar to that seen with wild-type HA. However, a slightly higher proportion of the HA-TM27 molecules were found in the aqueous supernatant indicating that removal of the cytoplasmic tail had slightly decreased the interaction of the protein with TX-114. Inspection of the results obtained with HA-TM17, HA-TM14, and HA-TM9 revealed that progressive truncation of

the transmembrane domain further decreased the ability of the mutant proteins to interact with the detergent. Nevertheless, >50% of the HA-TM17 or HA-TM14 molecules partitioned into TX-114 suggesting that these truncated molecules still interact significantly with the detergent, and by analogy, with the lipid bilayer. Truncation of the transmembrane domain to 9 amino acids resulted in the partitioning of the great majority of the HA-TM9 molecules into the aqueous phase. However, the small but significant proportion of the mutant found in the detergent phase (compared to the total absence of the X-31 HA-A<sup>-</sup> in this phase) suggests that HA-TM9 may still interact weakly with the lipid bilayer.

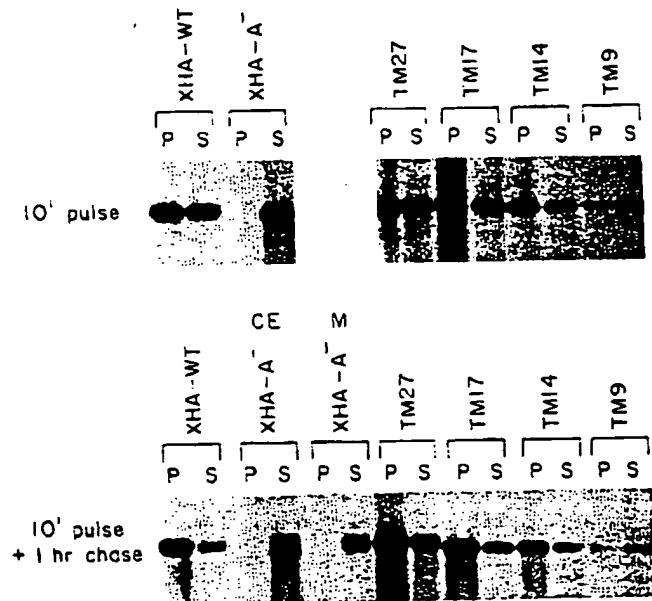
### Discussion

We have analyzed the biosynthesis and intracellular transport of a series of mutants of influenza virus hemagglutinin that express truncated forms of the protein. The mutants code for



**Figure 6.** Tryptic cleavage of wild-type X-31 HA and HA-TM17 expressed at the cell surface. (A) CV-1 cells infected for 42 h with SVEXHA or SVEXHA-TM17 recombinant viruses were labeled for 10 min with [<sup>35</sup>S]methionine and then incubated in medium containing an excess of nonradioactive methionine for the times shown. During the last 15 min of the chase period, trypsin (5  $\mu$ g/ml) was added to the medium above the monolayer. Cell extracts were then prepared, and proteins were immunoprecipitated with anti-HA serum, separated by SDS PAGE, and autoradiographed. (B) Longer exposure of the same autoradiogram showing the small amount of HA2 that is generated by tryptic cleavage of HA-TM17 at the cell surface.

HAs that lack the short carboxy-terminal hydrophilic tail but retain some (HA-TM9, -TM14, and -TM17) or all (HA-TM27) of the hydrophobic amino acids that normally anchor the protein in the lipid bilayer. A summary of the properties of



**Figure 7.** Triton X-114 partitioning of wild-type and truncated X-31 HA proteins. CV-1 cells infected for 42 h with SVEXHA, SVEXHA-A<sup>-</sup>, or SVEXHA-TM recombinant virus stocks were labeled for 10 min with [<sup>35</sup>S]methionine and then incubated in medium containing an excess of nonradioactive methionine for 1 h. Cell extracts were prepared in buffer containing 1% Triton X-114, and the extracts (or the medium collected from above the cells infected with SVEXHA-A<sup>-</sup>) were partitioned as described in Materials and Methods. Equal aliquots of the detergent pellet (P) and aqueous supernatant (S) phases were immunoprecipitated with anti-HA serum. Aliquots of the precipitated proteins were then separated by SDS PAGE and autoradiographed.

the mutants is shown in Table I. These results suggest the following conclusions.

(a) The cytoplasmic tail is not needed for the efficient transport of HA to the cell surface. HA-TM27 retains an intact membrane-spanning domain of 27 hydrophobic amino acids but contains none of the hydrophilic amino acids that form the cytoplasmic tail of HA. Because the intracellular transport, stability, and biological properties of HA-TM27 are indistinguishable from those of wild-type HA, we conclude that the cytoplasmic tail contains no signals essential for transport of the protein along the exocytotic pathway. Although HA-TM27 is a mutant of the X-31 (Aichi) strain of influenza virus, similar results have been obtained with a corresponding tail-minus mutant of the HA of the Japan strain of the virus (our unpublished results). We have previously shown that some but not all alterations to the cytoplasmic tail of the Japan HA affect the efficiency with which the protein is transported to the cell surface (12). These results could be best explained in terms of malfolding of the affected molecules: deleterious alterations to the cytoplasmic domain were postulated to destabilize the trimeric hemagglutinin or to delay or prevent its assembly. Molecules with such structural defects might be expected not to travel to the cell surface.

**Table I.** Summary of the Intracellular Transport of Wild-type and Truncated HA Proteins

	Translocation to ER*	Transport to Golgi	Intracellular location
HA-X31-WT	+	30 min	100% surface
HA-TM27	+	30 min	100% surface
HA-TM17	+	6-8 h (5-10%)	~20% surface ~80% ER, Golgi
HA-TM14	+	-	ER
HA-TM9	+	-	ER Lysosome?

\* ER, endoplasmic reticulum.

as efficiently as wild-type HA. The results presented here add strength to this hypothesis since they eliminate the possibility that the tail plays an active role in transport of HA along the exocytic pathway. Furthermore, the hypothesis can easily accommodate the observation that HA is less tolerant of alterations to the cytoplasmic tail than of its complete removal. We have recently shown (18a) that a mutant HA (HAxpBR; reference 12) which has an extended cytoplasmic tail, and whose intracellular transport is blocked in the endoplasmic reticulum, is malfolded and does not form the correct trimeric structure (55) of wild-type HA.

The effects of alterations to the cytoplasmic tails of several other transmembrane proteins have recently been analyzed. Zuniga et al. (64) have shown that removal of the tail from the murine histocompatibility antigen H2 did not necessarily prevent expression of the protein on the surface of cells. Similar results have been obtained with mutants of Semliki Forest virus E2 protein (6, 14). Furthermore, a natural variant of the low density lipoprotein receptor has been isolated that lacks all but 2 of the 50-amino acid cytoplasmic tail of the molecule (28). While this molecule is unable to undergo endocytosis, it is transported efficiently to the plasma membrane although at half the rate of the wild-type molecule. The conclusion drawn from these studies is that although the cytoplasmic tails of integral membrane proteins may be involved in endocytosis (28, 44), they play no essential role in exocytosis. A different view has been expressed by Rose and his co-workers, who have shown that mutation or substitution of the cytoplasmic tail of VSV G protein can prevent or retard the transport of the protein along the exocytic pathway (37, 43). They have proposed that the cytoplasmic tail of G protein carries a positive signal which controls the movement of the molecule to the cell surface. However, an alternative explanation would be that alteration of the cytoplasmic tail could affect the transport of G protein by affecting its structure. Analysis of the transport of a mutant which lacks all sequences of the cytoplasmic tail without any additional foreign residues might resolve this issue.

(b) The "stop-transfer" sequences are probably entirely contained within the hydrophobic transmembrane domain of HA. The cytoplasmic tails of most transmembrane proteins contain one or more positively charged amino acids and it has been proposed that such amino acids, acting in conjunction with the adjacent hydrophobic domain, may prevent further translocation of the polypeptide across the membrane of the rough endoplasmic reticulum (45). The fact that complete removal of the cytoplasmic tail of either HA or H-2 (64) or removal of the basic residues from the tail of SFV E2 (6) or from coliphage f1 gene III protein (9) does not convert these integral membrane proteins into secretory molecules argues that the charged amino acids are not essential components of the stop-transfer signal. However, removal of the cytoplasmic tail and all but the first two amino acids of the transmembrane domain did result in secretion of the truncated X-31 HA-A<sup>-</sup> molecule from the cell (see Figs. 3 and 7). It is likely therefore that the hydrophobicity of the transmembrane domain is both necessary and sufficient to prevent complete translocation of integral membrane proteins.

(c) 17 hydrophobic amino acids are sufficient to anchor HA in the lipid bilayer. Those molecules of HA-TM17 which reach the cell surface are not secreted but appear to be stably inserted in the plasma membrane, as monitored by binding

of erythrocytes to the small proportion of the protein that reaches the cell surface. The size of hydrophobic transmembrane domains has been thought to be constrained to a minimum of 20 amino acids by the need to span in an  $\alpha$ -helical conformation the 3-nm width of the hydrophobic core of the lipid bilayer (10, 13, 23, 34, 52). However, that the minimum size for a functional hydrophobic anchor is smaller than 20 residues is suggested by the observation of a 17-amino acid uncharged segment in the putative transmembrane domain of the T cell receptor  $\beta$ -subunit (22, 59). Furthermore, Davis and Model (8) and Davis et al. (9) have shown firstly that a mutant of the f1 gene III protein having a hydrophobic core of only 17 residues shows no impairment of anchor function and secondly that artificial domains of 16 or more hydrophobic amino acids function to anchor the gene III protein in the lipid bilayer. Adams and Rose (2) showed that VSV G proteins with truncated hydrophobic domains that are even shorter retain their transmembrane orientation. However, a possibility exists in these cases to bury an additional short hydrophobic stretch from the cytoplasmic domain into the lipid bilayer. Whether these shorter transmembrane domains do in fact cross the membrane in some form of helix remains to be determined.

(d) Severe truncation of the hydrophobic anchor results in HAs that are unstable and transported inefficiently along the exocytic pathway. Truncation of the hydrophobic anchor to 9 (HA-TM9) or 14 (HA-TM14) amino acids results in HAs whose transport appear to be blocked in the endoplasmic reticulum or in a pre-Golgi compartment. This phenotype is similar to that of mutants of VSV G protein in which the central region of the hydrophobic anchor has been either deleted (2) or disrupted by the insertion of a charged amino acid (1). Why truncation of the anchor should affect transport of these proteins is unknown, although once again structural deformity of the molecule, or, in the case of HA, an inability to form stable trimers, would seem to be likely explanations.

Both HA-TM14 and HA-TM9 are considerably less stable than wild-type HA and other mutants of HA that are blocked in the endoplasmic reticulum (12). HA-TM9 is particularly sensitive to degradation and disappears from the cell within 2 h of synthesis. That the instability and lack of transport of these mutant HAs are not simply due to the extent of truncation is demonstrated by the expression and secretion from cells of stable ectodomain forms of X-31 HA (Fig. 7) or Japan HA (17) that lack their entire transmembrane domains and cytoplasmic tails. The mechanisms by which defective membrane-associated proteins are identified and eliminated are not known. There is evidence in other systems for the rapid turnover of cytoplasmic proteins—particularly those which are badly folded (e.g., proteins synthesized in the presence of amino acid analogues) (24). Lysosomal and nonlysosomal mechanisms, including the ATP-dependent ubiquitin-mediated pathway, have been implicated in these processes. However, there is as yet no evidence for a similar system to scavenge defective membrane or secretory proteins that are trapped in the exocytic pathway. Although the intracellular site at which HA-TM9 and -TM14 are degraded is unknown, two lines of circumstantial evidence indicate that lysosomes are not involved. First, the core oligosaccharide groups of HA-TM9 and -14 show no evidence of the trimming and processing reactions that would be expected to occur as the proteins pass through the Golgi apparatus on the way to

the lysosome, and second, incubation of cells expressing HA-TM9 in the presence of chloroquine (a lysosomotropic agent that raises the pH of lysosomes and thereby blocks proteolysis in those organelles [49]) does not inhibit degradation of the protein.

Our current working hypothesis is that the fate of membrane proteins is determined by events occurring during or soon after the nascent polypeptides are translocated to the luminal face of the rough endoplasmic reticulum. If the protein can fold correctly, it will move from the endoplasmic reticulum and travel along the exocytic pathway to the Golgi apparatus. If it is malformed, the protein may become permanently trapped in the endoplasmic reticulum or it may be transported more slowly than usual along the pathway. In the most extreme case, presumably when its structure is grossly deranged, the protein is degraded. The results described in this paper are consistent with the likelihood that alterations in the transmembrane domain of HA can inhibit the correct folding of the molecule, possibly by interfering with the stacking of the subunits to form the HA trimer. We have recently analyzed the folding pathways of both wild-type and mutant forms of HA and have obtained evidence supporting this hypothesis that correct folding and trimerization is a prerequisite for intracellular transport (18a). Our future work will be directed towards investigating the mechanisms by which mammalian cells monitor whether membrane proteins are correctly folded. The best hope of finding an answer to this question seems to lie in analyzing the properties and functions of cellular proteins resident in the endoplasmic reticulum that interact with nascent secretory and membrane proteins.

This work was supported by grants from the National Institutes of Health and the American Cancer Society to M.-J. Gething and J. Sambrook.

Received for publication 24 April 1986, and in revised form 11 June 1986.

## References

1. Adams, G. A., and J. K. Rose. 1985. Incorporation of a charged amino acid into the membrane-spanning domain blocks cell surface transport but not membrane anchoring of a viral glycoprotein. *Mol. Cell. Biol.* 5:1442-1448.
2. Adams, G. A., and J. K. Rose. 1985. Structural requirements of a membrane-spanning domain for protein and cell surface transport. *Cell.* 41:1007-1015.
3. Alcaraz, G., J.-P. Kinet, N. Kumar, S. A. Wank, and H. Metzger. 1984. Phase separation of the receptor for immunoglobulin E and its subunits in Triton X-114. *J. Biol. Chem.* 259:14922-14927.
4. Blobel, G. 1980. Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA.* 77:1496-1500.
5. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604-1607.
6. Cutler, D. F., and H. Garoff. 1986. Mutants of the membrane-binding region of Semliki Forest Virus E2 protein. I. Cell surface transport and fusogenic activity. *J. Cell Biol.* 102:889-901.
7. Cutler, D. F., P. Melancon, and H. Garoff. 1986. Mutants of the membrane-binding region of Semliki Forest Virus E2 protein. II. Topology and membrane binding. *J. Cell Biol.* 102:902-910.
8. Davis, N. G., and P. Model. 1985. An artificial anchor domain: hydrophobicity suffices to stop transfer. *Cell.* 41:607-614.
9. Davis, N. G., J. D. Boeke, and P. Model. 1985. Fine structure of a membrane anchor domain. *J. Mol. Biol.* 181:111-121.
10. Deisenhofer, J., O. Epp, K. Miki, R. Huber, and H. Michel. 1985. Structure of the protein subunits in the photosynthetic reaction center of *Rhodopseudomonas viridis* at 3 Å resolution. *Nature (Lond.)*, 318:618-624.
11. Doms, R. W., M.-J. Gething, J. Henneberry, J. White, and A. Helenius. 1986. Variant influenza virus hemagglutinin that induces fusion at elevated pH. *J. Virol.* 57:603-613.
12. Doyle, C., M. G. Roth, J. Sambrook, and M.-J. Gething. 1985. Mutations in the cytoplasmic domain of the influenza hemagglutinin affect different stages of intracellular transport. *J. Cell Biol.* 100:704-714.
13. Eisenberg, D. 1984. Three-dimensional structure of membrane and surface proteins. *Annu. Rev. Biochem.* 53:595-623.
14. Garoff, H., C. Kondor-Koch, R. Pettersson, and B. Burke. 1983. Expression of Semliki Forest virus proteins from cloned complementary DNA. II. The membrane-spanning glycoprotein E2 is transported without its normal cytoplasmic domain. *J. Cell Biol.* 97:652-658.
15. Garten, W., F.-X. Bosch, D. Linder, R. Roth, and H.-D. Klenk. 1981. Proteolytic activation of the influenza virus hemagglutinin: the structure of the cleavage site and the enzymes involved in cleavage. *Virology*, 115:361-374.
16. Gething, M.-J., and J. Sambrook. 1981. Cell-surface expression of influenza haemagglutinin from a cloned DNA copy of the RNA gene. *Nature (Lond.)*, 293:620-625.
17. Gething, M.-J., and J. Sambrook. 1982. Construction of influenza haemagglutinin genes that code for intracellular and secreted forms of the protein. *Nature (Lond.)*, 300:598-603.
18. Gething, M.-J., C. Doyle, M. G. Roth, and J. Sambrook. 1985. Mutational analysis of the influenza hemagglutinin. *Curr. Top. Membr. Transp.* 23:17-41.
- 18a. Gething, M.-J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell.* In press.
19. Gluzman, Y. 1981. SV40 transformed simian cells support the replication of early SV40 mutants. *Cell.* 23:177-182.
20. Guan, J.-L., and J. K. Rose. 1984. Conversion of a secretory protein into a transmembrane protein results in its transport to the Golgi complex but not to the cell surface. *Cell.* 37:779-785.
21. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557-580.
22. Hedrick, S., E. A. Neilsen, J. Kavalier, D. I. Cohen, and M. Davis. 1984. Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins. *Nature (Lond.)*, 308:153-158.
23. Henderson, R., and P. N. T. Unwin. 1975. Three-dimensional model of purple membrane obtained by electron microscopy. *Nature (Lond.)*, 257:28-32.
24. Hershko, A., and A. Ciehanover. 1982. Mechanisms of intracellular protein breakdown. *Annu. Rev. Biochem.* 51:335-364.
25. Kilbourne, E. D. 1969. Future influenza vaccines and the use of genetic recombinants. *Bull. W.H.O.* 41:643-645.
26. Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631-664.
27. Lasky, L. A., D. Dowbenko, C. C. Simonsen, and P. W. Berman. 1984. Protection of mice from lethal herpes simplex virus infection by vaccination with a secreted form of cloned glycoprotein D. *BioTechnology*, 2:527.
28. Lehrman, M. A., J. L. Goldstein, M. S. Brown, D. W. Russell, and W. J. Schneider. 1985. Internalization-defective LDL receptors produced by genes with nonsense and frameshift mutations that truncate the cytoplasmic domain. *Cell.* 41:735-743.
29. Louvard, D., H. Reggio, and G. Warren. 1982. Antibodies to the Golgi complex and the rough endoplasmic reticulum. *J. Cell Biol.* 92:92-107.
30. Maniatis, T., E. Fritsch, and J. Sambrook. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
31. Messing, J., and J. Vieira. 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. *Gene.* 19:269-276.
32. Murre, C., C. S. Reiss, C. Bernabeu, L. B. Chen, S. J. Burakoff, and J. G. Seidman. 1984. Construction, expression, and recognition of an H-2 molecule lacking its carboxyl terminus. *Nature (Lond.)*, 307:432-436.
33. Nakamura, K., and R. W. Compans. 1979. Host cell- and virus strain-dependent differences in oligosaccharides of hemagglutinin glycoproteins of influenza A viruses. *Virology*, 95:8-23.
34. Noda, M., S. Shimizu, T. Tanabe, T. Takai, T. Kayano, T. Ikeda, H. Takahashi, H. Nakayama, Y. Kanazawa, N. Minamino, K. Kangawa, H. Matsuo, M. A. Raftery, T. Hirose, S. Inayama, H. Hayashida, T. Miyata, and S. Numa. 1984. Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence. *Nature (Lond.)*, 312:121-127.
35. Owen, M. J., A.-M. Kissnerghis, and H. F. Lodish. 1980. Biosynthesis of HLA-A and HLA-B antigens in vivo. *J. Biol. Chem.* 255:9678-9684.
36. Poruchinsky, M. S., C. Tyndall, G. W. Both, F. Sato, A. R. Bellaray, and P. H. Atkinson. 1985. Deletions into an NH<sub>2</sub>-terminal hydrophobic domain result in secretion of rotavirus VP7, a resident endoplasmic reticulum membrane glycoprotein. *J. Cell Biol.* 101:2199-2209.
37. Puddington, L., C. E. Machamer, and J. K. Rose. 1986. Cytoplasmic domains of cellular and viral integral membrane proteins substitute for the cytoplasmic domain of the vesicular stomatitis virus glycoprotein in transport to the plasma membrane. *J. Cell Biol.* 102:2147-2157.
38. Reidel, H. 1985. Different membrane anchors allow the Semliki Forest Virus spike subunit E2 to reach the cell surface. *J. Virol.* 54:224-228.
39. Rettenmier, C. W., M. F. Roussel, C. O. Quinn, G. R. Kitchingman, A. T. Loo, and C. J. Sherr. 1985. Transmembrane orientation of glycoproteins encoded by the v-fms oncogene. *Cell.* 40:971-981.
40. Rizzolo, L. J., J. Finidori, A. Gonzalez, M. Arpin, I. E. Ivanov, M. Adesnik, and D. D. Sabatini. 1985. Biosynthesis and intracellular sorting of growth hormone-viral envelope glycoprotein hybrids. *J. Cell Biol.* 101:1351-1362.
41. Rogers, J., P. Early, C. Carter, K. Calame, M. Bond, L. Hood, and R. Wall. 1980. Two mRNAs with different 3' ends encode membrane-bound and secreted forms of immunoglobulin u chain. *Cell.* 20:303-312.

42. Rose, J. K., and J. E. Bergmann. 1982. Expression from cloned cDNA of cell surface and secreted forms of the glycoprotein of vesicular stomatitis virus in eukaryotic cells. *Cell*. 30:753-762.

43. Rose, J. K., and J. E. Bergmann. 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. *Cell*. 34:513-524.

44. Roth, M. G., C. Doyle, J. Sambrook, and M.-J. Gething. 1986. Heterologous transmembrane and cytoplasmic domains direct functional chimeric influenza virus hemagglutinins into the endocytic pathway. *J. Cell Biol.* 102: 1271-1283.

45. Sabatini, D., G. Kreibich, T. Morimoto, and M. Adesnik. 1982. Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* 92:1-22.

46. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*. 74:5463-5467.

47. Sanger, F., A. R. Coulson, B. G. Barrell, A. J. H. Smith, and B. A. Roe. 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* 143:161-178.

48. Saraste, J., and E. Kuismaen. 1984. Pre- and post-Golgi vesicles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell*. 38:538-549.

49. Seglen, P. O. 1983. Inhibitors of lysosomal function. *Methods Enzymol.* 9:737-765.

50. Skehel, J. S., and M. D. Waterfield. 1975. Studies on the primary structure of the influenza virus haemagglutinin. *Proc. Natl. Acad. Sci. USA*. 72: 93-97.

51. Sveda, M. S., L. J. Markoff, and C.-J. Lai. 1982. Influenza virus hemagglutinin containing an altered hydrophobic carboxy terminus accumulates intracellularly. *J. Virol.* 49:223-228.

52. Tanford, C. 1978. The hydrophobic effect and the organization of living matter. *Science (Wash. DC)*. 200:1012-1018.

53. Tarentino, A. L., and F. Matey. 1974. Purification and properties of an endo-B-acetylglucosaminidase from *Streptomyces griseus*. *Mol. Cell. Biol.* 4:688-694.

54. Warren, G. 1981. In *Membrane Structure*. J. B. Finean and R. H. Mitchell, editors. Elsevier/North Holland Biomedical Press, Amsterdam. 215-257.

55. Wiley, D. C., J. J. Skehel, and M. D. Waterfield. 1977. Evidence from studies with a cross-linking reagent that the hemagglutinin of influenza virus is a trimer. *Virology*. 79:446-448.

56. Wills, J. W., J. M. Hardwick, K. Shaw, and E. Hunter. 1983. Alterations in the transport and processing of Rous Sarcoma virus envelope glycoproteins mutated in the signal and anchor regions. *J. Cell. Biochem.* 23:81-94.

57. Wills, J. W., R. J. Srinivas, and E. Hunter. 1984. Mutations in the Rous Sarcoma virus *env* gene that affect the transport and subcellular location of the glycoprotein products. *J. Cell Biol.* 99:2011-2023.

58. Wilson, I. A., J. J. Skehel, and D. C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3A resolution. *Nature (Lond.)*. 289:366-378.

59. Yanagi, Y., Y. Yoshikai, K. Leggett, S. P. Clark, I. Aleksander, and T. Mak. 1984. A human T-cell specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature (Lond.)*. 308:146-149.

60. Yost, C. S., J. Hedgepeth, and V. R. Lingappa. 1983. A stop transfer sequence confers predictable transmembrane orientation to a previously secreted protein in cell-free systems. *Cell*. 34:759-766.

61. Zoller, M. J., and M. Smith. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 derived vectors. *Methods Enzymol.* 100:468-500.

62. Zoller, M. J., and M. Smith. 1985. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. *DNA*. 3:479-488.

63. Zuniga, M. C., B. Malissen, M. McMillan, P. R. Brayton, S. S. Clark, J. Forman, and L. Hood. 1983. Expression and function of transplantation antigens with altered or deleted cytoplasmic domains. *Cell*. 3:535-544.

64. Zuniga, M. C., and L. E. Hood. 1986. Clonal variation in cell surface display of an H-2 protein lacking a cytoplasmic tail. *J. Cell Biol.* 102:1-10.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**  
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.